Saskatchewan

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Agriculture Development Fund

FINAL REPORT

VACCINE POTENTIAL OF BACTERIAL HEAT SHOCK PROTEINS

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Vaccine potential of bacterial heat shock proteins

Project #96000255

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A. SUMMARY

The objective of this research program was to test the utility of *Haemophilus somnus* heat shock proteins as vaccine components as well as to test their use as an adjuvant for the production of other veterinary vaccine components. The latter objective included the ability of heat shock proteins to aid in the formulation of soluble recombinant proteins as well as an analysis of their ability to boost the immune response to other vaccine antigens. Animals vaccinated with the two heat shock proteins, GroEL and DnaK, alone or in combination, were not significantly protected against experimental infection with virulent *H. somnus*. Thus, they do not appear to have any utility as vaccine components. The heat shock proteins were able to aid in the formulation of other recombinant proteins, but not at a level sufficient to make them commercially useful. DnaK was able to boost the immune response to leukotoxin in mice, indicating that it may be a useful component of subunit vaccines. We are pursuing this possibility further in cattle.

B. EXECUTIVE SUMMARY

Haemophilus somnus is a major cause of economic loss to beef producers in Canada and although vaccines have been available for a number of years, it is difficult to completely prevent infection in feedlots. The bacteria is able to evade the primary line of defence in the animal, namely white blood cells, by being able to grow in these cells. This property is typical of many bacterial pathogens and it has been shown with other species of bacteria that vaccination with heat-shock proteins, which are produced in response to stress, can eliminate colonization by intracellular pathogens. Heat shock proteins also have been shown to be useful in the production of vaccine components by ensuring that they are folded into the correct structure and remain soluble. In addition, when these proteins are mixed with other vaccine antigens, they have the potential to stimulate immune responses in the absence of traditional mineral oil-based adjuvants. Therefore, heat shock proteins are potentially useful not only as vaccine components themselves but for the production of other vaccine components and as adjuvants. The objectives of this research project were to test the utility of the *H. somnus* heat shock proteins GroEL and DnaK in each of these three areas.

A vaccine trial was conducted in which cattle were immunized with 100 μg of recombinant GroEL or DnaK, alone and in combination, with a mineral oil adjuvant (VSA3). After two immunizations, animals were challenged with bovine herpesvirus-1 followed by virulent *H. somnus*. Animals were monitored daily for signs of disease and it became apparent after eight days that vaccination had no beneficial effect compared to a placebo group and thus all animals were treated with antibiotics.

The ability of GroEL and DnaK to aid in the production of other vaccine antigens was tested by combining the genes coding for these proteins with the genes coding for the *Pasteurella haemolytica* leukotoxin as well as the *H. somnus* transferrin-binding protein Tbp2. These genes were assembled in such a fashion that they either expressed each protein independently or as a single fusion protein. Since the leukotoxin and transferrin-binding protein Tbp2 are normally insoluble in *E. coli*, we measured the ability of GroEL to drive the expression of the proteins to a soluble form. The DnaK fusions were lethal to the cell and thus could not be tested. GroEL was able to drive the expression of the *P. haemolytica* leukotoxin to a soluble form whether it was

expressed as a fusion protein or independently from the leukotoxin. However, the amount of soluble protein produced was not sufficient to make vaccine production economical by this route.

Finally, both GroEL and DnaK were tested for their adjuvant activity, utilizing the *P. haemolytica* leukotoxin as a model antigen. Groups of 15 mice were immunized with various combinations of GroEL and leukotoxin or DnaK and leukotoxin and the serological response to vaccination was measured at the time of each vaccination (21 days apart) and ten days following the second immunization. In addition, antigen-specific cytokine production (interleukin-4 and interferon-γ) by splenocytes was measured at the end of the trial. The addition of GroEL in any form did not result in stimulation of immune responses to the *P. haemolytica* leukotoxin while addition of DnaK did result in both higher antibody levels following both immunizations as well as an increase in interleukin-4 production. Therefore, we feel that DnaK from *H. somnus* may have utility as an adjuvant in cattle and we plan on following this work up with studies combining DnaK with other antigens currently under development for bovine vaccines.

C. TECHNICAL REPORT

1. Background

Heat shock proteins are produced by all organisms in response to stress and they play a major role in ensuring that proteins produced in the cell are folded into the appropriate structure. In the case of bacteria, they have also been shown to be major targets for the immune system following infection, and in some cases, they appear to be protective antigens (eg. mycobacteria). In addition to the above, heat shock proteins have the ability in some cases to enhance the immune response to other antigens (adjuvant activity). Therefore, bacterial heat shock proteins have three important functions which can be exploited for use in the animal health field, namely, a) vaccine development, b) formulation of recombinant veterinary vaccines, and c) development of novel adjuvants to increase the immune response to existing vaccines.

Haemophilus somnus has re-emerged as an important disease in Western Canada and is now the most economically important pathogen of feedlot cattle. It is also part of the bovine respiratory disease complex which costs North American cattle producers up to $1 Billion annually in lost revenue. *H. somnus* is capable of surviving in white blood cells, the first line of defence against bacterial infection, and therefore it would be useful to develop vaccination strategies which would protect against this property of the organism. Vaccination with heat shock proteins (GroEL and DnaK) has been shown to reduce intracellular survival with some other pathogens and thus the primary objective of this project was to test their vaccine potential against experimental *H. somnus* infection.

2. Objectives

This project had three major objectives, including:

a) to determine the protective capacity of *H. somnus* GroEL and DnaK proteins, alone and in combination, against experimental *H. somnus* infection.

b) To test the ability of recombinant heat shock proteins to aid in the production of other recombinant antigens in a soluble form.

c) To test the ability of GroEL and DnaK to increase the immune response to other vaccine antigens, including the *P. haemolytica* leukotoxin and *H. somnus* transferrin-binding protein.

3. Results

Objective 1: Protective capacity of *H. somnus* GroEL and DnaK.

We have characterized two *H. somnus* heat shock proteins called GroEL and DnaK and have isolated and expressed the genes at high levels in *E. coli*. Unlike many recombinant proteins, these can be recovered from *E. coli* in a soluble form and therefore are easily purified using conventional biochemical techniques. These two recombinant proteins were purified by ion exchange chromatography and four different vaccines were formulated such that each contained 100 μg of protein combined with the adjuvant VSA3. The four vaccines contained GroEL (group 1), DnaK (group 2), placebo (group 3) or GroEL + DnaK (group 4). The placebo consisted of the adjuvant VSA3 combined with sterile saline. Groups of ten calves were vaccinated subcutaneously with 2 cc of each vaccine and three weeks later, all groups were boosted with the appropriate formulation. Two weeks after the second immunization, animals were challenged with bovine herpesvirus-1 followed four days later by an aerosol of virulent *H. somnus*. All animals were examined daily throughout the trial by a veterinarian and animal health technicians and various parameters, including temperature, nasal scores, depression, strength, respiratory distress, overall sick scores and weight loss were recorded daily. The clinical results are summarized in Table 1 showing the average group scores in each category for each day of the trial and it can be seen that there is no significant difference between any of the groups. The raw data is attached in Appendix 1. Therefore, unlike other diseases caused by intracellular pathogens, it appears as if the *H. somnus* heat shock protein are not effective vaccine components when animals are exposed to the organism by the respiratory route. No differences were observed in the serological response to immunization in any of the three groups receiving *H. somnus* antigen and thus the lack of protection cannot be explained by a poor response to the vaccine.

Table 1: Clinical Scores

Group	Day	Temperature	Nasal	Depression	Strength	Respiratory	Sick	Survivors
GroEL	0	38.51	0.00	0.00	0.00	0.00	0.00	1.00
DnaK	0	38.53	0.00	0.06	0.06	0.00	0.00	1.00
Placebo	0	38.33	0.00	0.00	0.00	0.00	0.00	1.00
GroEL + DnaK	0	38.45	0.00	0.00	0.00	0.00	0.00	1.00
GroEL	1	38.60	0.00	0.00	0.00	0.00	0.00	1.00
DnaK	1	38.58	0.00	0.06	0.06	0.00	0.06	1.00
Placebo	1	38.44	0.00	0.00	0.00	0.00	0.00	1.00
GroEL + DnaK	1	38.29	0.00	0.00	0.00	0.00	0.00	1.00
GroEL	2	40.63	0.05	0.00	0.00	0.00	0.00	1.00
DnaK	2	40.32	0.06	0.06	0.06	0.00	0.06	1.00
Placebo	2	40.58	0.05	0.00	0.00	0.00	0.00	1.00
GroEL + DnaK	2	40.35	0.00	0.00	0.00	0.00	0.00	1.00
GroEL	3	40.27	0.60	0.00	0.00	0.00	0.30	1.00
DnaK	3	40.07	0.56	0.00	0.00	0.00	0.11	1.00
Placebo	3	40.34	0.80	0.00	0.00	0.00	0.20	1.00
GroEL + DnaK	3	40.17	0.50	0.05	0.00	0.00	0.15	1.00
GroEL	4	40.48	1.55	0.00	0.00	0.00	0.30	1.00
DnaK	4	40.52	1.39	0.06	0.00	0.00	0.39	1.00
Placebo	4	40.55	1.61	0.00	0.00	0.00	0.40	1.00
GroEL + DnaK	4	40.66	1.35	0.00	0.00	0.00	0.40	1.00
GroEL	5	40.51	1.70	0.90	0.00	0.10	1.20	1.00
DnaK	5	40.48	1.72	0.88	0.25	0.44	1.06	1.00
Placebo	5	40.64	1.90	0.70	0.00	0.10	0.80	1.00
GroEL + DnaK	5	39.88	1.35	0.90	0.30	0.30	0.85	1.00
GroEL	6	39.48	1.79	1.75	1.38	1.38	2.06	1.00
DnaK	6	40.28	1.17	1.88	1.29	1.29	1.88	1.00
Placebo	6	40.74	1.75	1.29	0.33	0.33	1.25	1.00
GroEL + DnaK	6	39.67	2.00	0.75	0.71	0.75	1.56	0.90
GroEL	7	40.27	1.50	1.60	1.40	1.00	1.80	0.50
DnaK	7	39.76	1.70	1.40	1.20	0.20	1.10	0.67
Placebo	7	40.21	1.57	1.17	1.00	0.33	1.00	0.90
GroEL + DnaK	7	39.43	1.33	0.83	0.33	0.00	0.67	0.70
GroEL	8	38.80	1.33	0.00	0.00	0.00	0.17	0.30
DnaK	8	39.20	1.67	1.67	0.40	0.17	1.25	0.67
Placebo	8	39.78	1.33	0.75	0.00	0.00	0.58	0.70
GroEL + DnaK	8	39.47	1.25	0.58	0.00	0.00	0.42	0.60

Objective 2: Use of heat shock proteins in gene expression systems.

Most recombinant veterinary vaccines currently available utilize antigens produced in *E. coli* as insoluble proteins known as inclusion bodies. The processing of these inclusion bodies can add cost to the manufacturing process and uses harsh chemicals such as guanidine hydrochloride to render the antigens soluble. The use of such chemicals can adversely affect meat quality if they are present at high concentrations in the final vaccine. Therefore, we tested the ability of the heat shock proteins GroEL and DnaK to drive recombinant protein production into a soluble form which can then be easily processed for use in vaccines. The antigens which we utilized for this study were the *P. haemolytica* leukotoxin and the *H. somnus* transferrin-binding protein Tbp2. These were chosen since both are protective antigens from pathogens involved in bovine respiratory disease and both are currently produced as inclusion bodies for vaccine formulation.

In order to carry out these experiments, we recloned the genes coding for GroEL, DnaK, *P. haemolytica* leukotoxin (lktA) and *H. somnus* transferrin-binding protein Tbp2 such that the latter genes were either co-expressed with the heat shock proteins in the same cell or were genetically fused to the heat shock proteins to produce a single antigen. These are outlined in Figure 1. The expression of each of these genetic constructs was inducible by the addition of lactose analogs to the bacterial growth medium, allowing us to carefully monitor the production of each protein. The levels of expression of each protein were monitored by a combination of polyacrylamide gel electrophoresis as well as reaction with specific antibody against each protein.

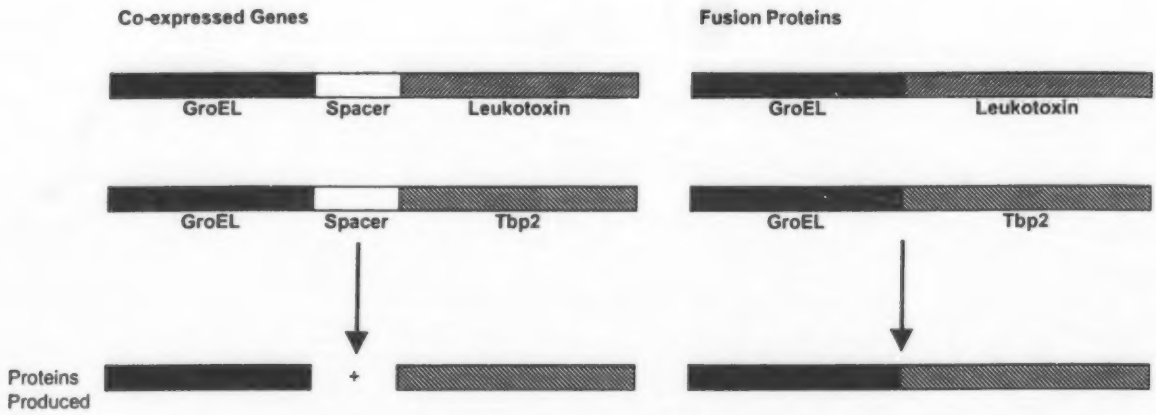

Figure 1: Structure of heat shock protein-antigen gene fusions and the proteins produced. In the case of co-expression of GroEL and either leukotoxin or Tbp2, a small spacer sequence was inserted between the two genes such that two separate proteins were produced in the same cell. In the case of fusion proteins, the spacer was omitted such that a fusion protein consisting of both GroEL and either leukotoxin or Tbp2 sequences was produced.

While GroEL fusions with each antigen were obtained, it was not possible to obtain fusions to the DnaK protein due to lethality of the recombinant constructs in the *E. coli*. While this could not be predicted at the start of this work, it is a relatively common occurrence when dealing with the co-expression of two different genes at high levels. Since both the leukotoxin and Tbp2 are proteins that are exported from the bacterial cell, it is likely that driving the expression to a soluble form would be toxic due to insertion of these proteins in the cell membrane. Therefore, our work focused on the ability of GroEL to drive the expression of leukotoxin and Tbp2 to the soluble form. Figures 2 and 3 illustrate Western Blots where soluble and insoluble fractions containing the GroEL::LKT fusion protein as well as the GroEL LKT co-expressed proteins were separated by electrophoresis and reacted with antibodies. The antisera used included mouse antibody specific for GroEL (Figure 2) as well as a monoclonal antibody specific for leukotoxin (Figure 3). It can be seen that the anti-GroEL antibody reacted with both high and low molecular weight forms of the fusion protein as well as the co-expressed protein (lanes 1-4) which indicated that there was some soluble GroEL::LKT protein being made. However, most was found in the insoluble fraction. Interestingly, in the co-expressed construct, a band with the same molecular weight as the fusion protein was observed, indicating that the GroEL and leukotoxin proteins formed a stable complex which could not be resolved by electrophoresis. GroEL is known to form complexes with proteins in the cell and thus this is

direct evidence that it was functional in this study. It can be seen in Figure 3 that the leukotoxin was expressed primarily in the insoluble form as well and interestingly, the monoclonal antibody did not react with the fusion protein but rather only with the native co-expressed LKT. When this was repeated using bovine convalescent serum against *P. haemolytica*, a band of the appropriate molecular weight was observed (Figure 4) indicating that the fusion protein was present but the leukotoxin epitope recognized by the monoclonal antibody was in a confirmationally-different form.

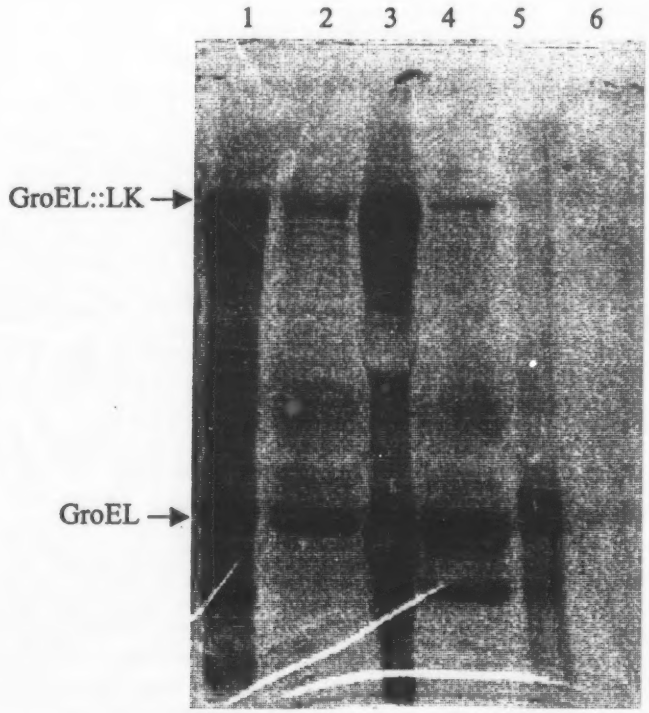

Figure 2: Reactivity of GroEL::LKT fusions and co-expressed proteins with anti-GroEL antibody. Whole cell lysates were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose by electroblotting and reacted with anti-GroEL serum. The arrow show the two primary bands containing GroEL, namely the fusion protein as well as the native GroEL protein. Lane 1, GroEL::LKT fusion insoluble fraction; Lane 2, GroEL::LKT fusion soluble fraction; Lane 3, GroEL-LKT coexpression insoluble fraction; Lane 4, GroEL-LKT co-expressed soluble fraction; Lane 5, pure GroEL protein; Lane 6, pure leukotoxin protein.

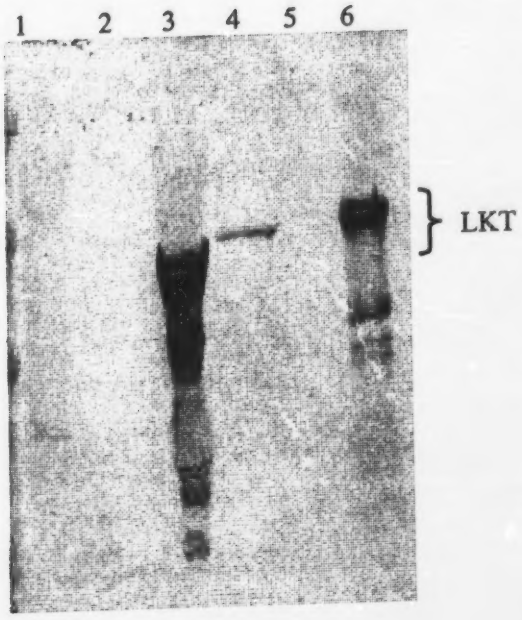

Figure 3: Reactivity of GroEL::LKT fusion proteins and co-expressed proteins with anti-leukotoxin monoclonal antibody. The order of samples is the same as in Figure 2. Note that this monoclonal antibody reacted only with the co-expressed GroEL-LKT sample and not the fusion protein (Lanes 1 and 2).

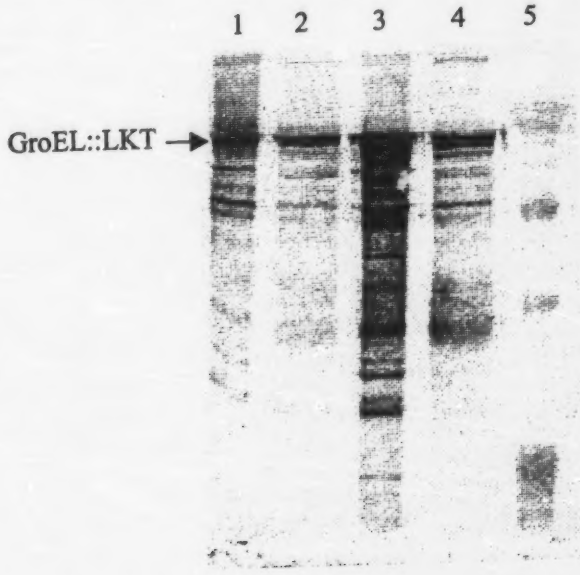

Figure 4: Reactivity of the GroEL::LKT fusion protein with bovine anti-*P. haemolytica* serum. Lanes 1-4 contain the GroEL::LKT fusion protein (two different preparations) with Lanes 1 and 3 representing insoluble material and Lanes 2 and 4 representing soluble material. The fusion protein, marked with an arrow, did not react with the monoclonal antibody (Figure 3) but does react with this polyclonal antisera.

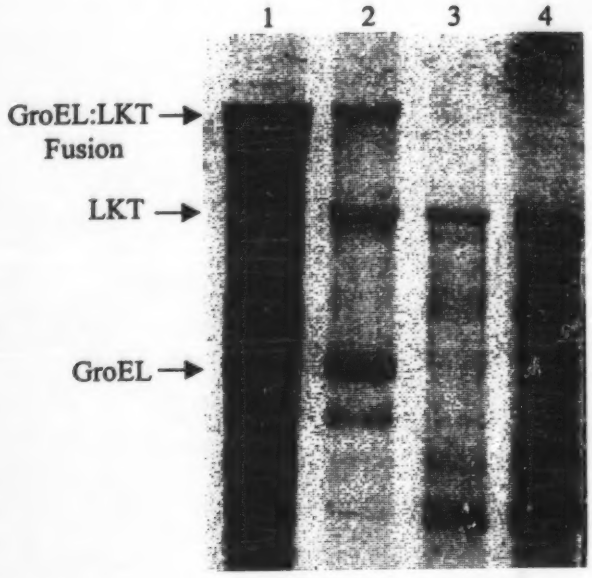

Figure 5: Polyacrylamide gel electrophoresis of GroEL::LKT fusion protein, GroEL-LKT, GroEL-LKT co-expressed protein and native LKT. Samples were separated by polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane 1, GroEL::LKT fusion protein; Lane 2, GroEL-LKT co-expressed proteins; Lane 3 and 4, LKT alone.

The results with the transferrin-binding protein Tbp2 were very similar and Figure 5 shows a GroEL::Tbp fusion separated by polyacrylamide gel electrophoresis. In this case, a protein band of approximately 125,000 corresponding to the fusion protein is observed in the insoluble fraction. Thus, we conclude that the co-expression of GroEL with leukotoxin or Tbp2 can result in a shift to soluble protein production, but the majority of the protein in the cell is still in the insoluble form and thus it would not be economical to produce vaccines in this fashion, since the two forms would have to be separated biochemically. Interestingly, during the latter stages of this work, a group in the United Kingdom published preliminary experiments using GroEL peptides from *E. coli* in which they were able to obtain efficient solubilization of recombinant proteins which could not be solubilized with the whole GroEL molecule. Thus, this may provide another avenue for the development of soluble protein expression systems in the future.

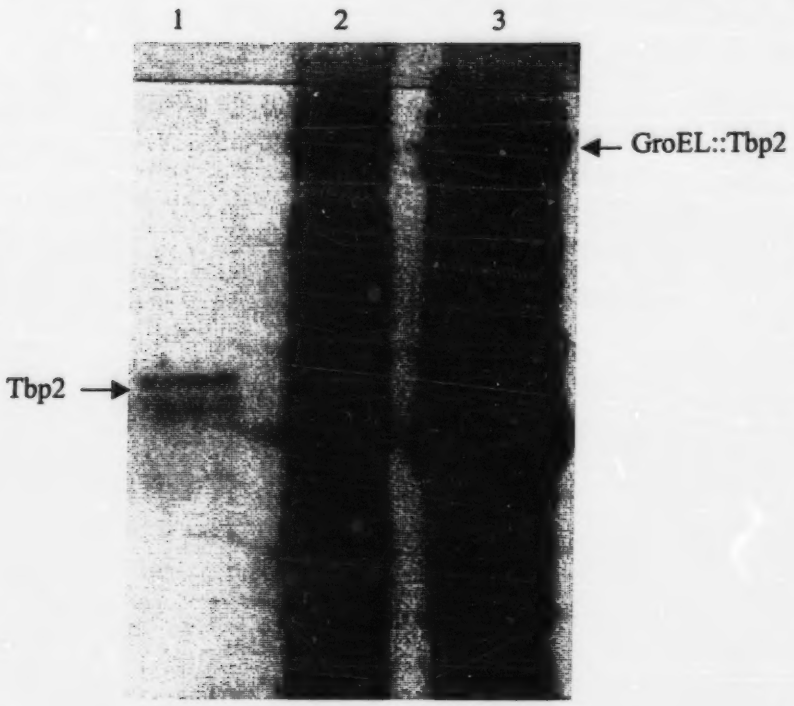

Figure 6: Polyacrylamide gel electrophoresis of the GroEL::Tbp2 fusion protein. Samples were separated by polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane 1, purified Tbp2; Lane 2, GroEL::Tbp2 uninduced cells; Lane 3, GroEL::Tbp2 induced cells. Note the presence of the fusion protein in Lane 3 (arrow).

Objective 3: Adjuvant activity of *H. somnus* heat shock proteins.

An experiment was carried out with the leukotoxin GroEL fusions as well as leukotoxin mixed with DnaK to measure the adjuvant activity of the two heat shock proteins. Nine groups of mice were immunized with 2 μg of leukotoxin, either alone or in combination with the molar equivalent of GroEL or DnaK as outlined in Table 2. The serological response to immunization was measured using an enzyme-linked immunosorbent assay and interferon-γ and interleukin-4 production by splenocytes was measured at the end of the experiment.

Table 2 shows the serological response to immunization using serum samples taken on the day of each immunization and ten days following the second immunization. Those mice which received the placebo (Group 9) showed high titers to the *P. haemolytica* leukotoxin indicating that they had been exposed to a cross-reactive antigen during the course of the trial. This is common since the leukotoxin is a member of the RTX toxin family which are produced by a wide range of Gram negative bacteria. Therefore, there was no significant serological response to immunization in any of the groups which did not receive a mineral oil-based

adjuvant. Group 8, which received leukotoxin formulated in VSA3, showed a significantly better response to both the primary and secondary immunizations. The group which received the DnaK protein mixed with leukotoxin (Group 6) also responded to the primary and secondary immunizations, although not as good as the group containing the mineral oil-based adjuvant and this is consistent with the results observed earlier with the DnaK fusion proteins which appear to be lethal to the bacterial cell. The lethality likely indicates biological activity and this is born out by the adjuvant activity seen in this experiment. Furthermore, this group also showed increased antigen-specific IL-4 production by spleen cells following the first immunization. This supports the conclusion that DnaK may be useful as an adjuvant for bovine respiratory disease vaccines. Therefore, we plan on pursuing DnaK as an adjuvant in cattle with other antigens.

Table 2: Serological Response to Vaccination

Group	Vaccine	Anti-Leukotoxin Serum Antibody		
		Prebleed	Day 21	Day 31
1	LKT	18	611	20240
2	GroEL::LKT Fusion	1229	1042	2250
3	GroEL + LKT Co-expressed	13	3372	19200
4	GroEL + LKT Mixed	266	4991	7169
5	DnaK	168	726	26148
6	DnaK + LKT Mixed	1804	11060	46367
7	GroEL	103	160	13623
8	LKT + Adjuvant (VSA3)	1432	32949	85350
9	Placebo (PBS)	71	18319	23989

Table 3: Antigen-Specific Cytokine Secretion by Mouse Splenocytes

Group	Vaccine	IL-4	IFN-γ
1	LKT	0	20.7 ± 2.8
2	GroEL::LKT Fusion	0	14 ± 2.2
3	GroEL + LKT Co-expressed	0	15 ± 2.4
4	GroEL + LKT Mixed	0	18 ± 3.8
5	DnaK	0	4.2 ± 2.2
6	DnaK + LKT Mixed	3 ± 2.2	11.7 ± 2.2
7	GroEL	0	0
8	LKT + Adjuvant (VSA3)	32.3 ± 3.6	66.7 ± 3.5
9	Placebo (PBS)		0

Results are number of positive cells per 5×10^5 spleen cells +/- the standard deviation. Five mice per group were used.

4. Conclusions

- Immunization of cattle with the *H. somnus* heat shock proteins GroEL and DnaK does not result in the induction of protective immunity against experimental challenge with *H. somnus*.
- The co-expression of GroEL with either the *P. haemolytica* leukotoxin or *H. somnus* transferrin binding protein Tbp2 does result in an increase in the amount of soluble protein available for purification. However, there is insufficient soluble protein made to make this a commercially viable process and it is likely that co-expression with subfragments of the GroEL protein would be an avenue to pursue in the future.
- While GroEL did not appear to exhibit any adjuvant activity with the *P. haemolytica* leukotoxin, the addition of DnaK to the leukotoxin did increase the serological response indicating this protein may be useful to pursue with respect to testing its effect on other antigens as well as its adjuvant properties in cattle.

D. PERSONNEL

Please refer to the Statement of Expenses.

E. EQUIPMENT

There was no equipment purchased on this grant.

APPENDIX 1

Calf #	Day	Wt. (kg)	Temp.	Nasal	Dep,	Str.	Resp.	Sick	Sex	Group
97-12	0	121	38.2	0	0	0	0	0	H	1
97-14	0	150	38.9	?	0	0	0	0	S	1
97-21	0	140	37.6	0	0	0	0	0	H	1
97-24	0	167	38.3	0	0	0	0	0	H	1
97-26	0	174	38.2	0	0	0	0	0	H	1
97-33	0	168	38.5	?	0	0	0	0	H	1
97-34	0	174	39.1	?	0	0	0	0	S	1
97-39	0	170	39.2	?	0	0	0	0	S	1
97-42	0	174	38.2	0	0	0	0	0	H	1
97-47	0	167	38.9	?	0	0	0	0	S	1
97-11	0	109	38.3	?	0	0	0	0	H	2
97-17	0	96		?	0.5	0.5	0	0	S	2
97-18	0	152	38.7	0	0	0	0	0	H	2
97-29	0	166	38.6	?	0	0	0	0	S	2
97-32	0	157	38.2	0	0	0	0	0	S	2
97-35	0	167	38.9	0	0	0	0	0	S	2
97-40	0	191	37.7	0	0	0	0	0	H	2
97-45	0	195	38.9	?	0	0	0	0	H	2
97-49	0	171	38.9	?	0	0	0	0	S	2
97-10	0	115	38.4	?	0	0	0	0	S	3
97-16	0	122	39.4	?	0	0	0	0	H	3
97-20	0	161	38.6	?	0	0	0	0	H	3
97-22	0	171	38.9	0	0	0	0	0	H	3
97-27	0	171	38.3	0	0	0	0	0	H	3
97-30	0	167	38	0	0	0	0	0	H	3
97-36	0	166	37.8	0	0	0	0	0	H	3
97-38	0	180	38.9	?	0	0	0	0	H	3
97-43	0	165	36.7	0	0	0	0	0	H	3
97-46	0	172	38.3	?	0	0	0	0	H	3
97-13	0	160	38.6	0	0	0	0	0	S	4
97-15	0	179	38.5	?	0	0	0	0	H	4
97-19	0	141	38.5	0	0	0	0	0	S	4
97-25	0	152	38.5	?	0	0	0	0	H	4
97-28	0	157	38.4	0	0	0	0	0	S	4
97-31	0	187	37.3	0	0	0	0	0	S	4
97-37	0	165	38.3	?	0	0	0	0	H	4
97-41	0	188	38.8	?	0	0	0	0	H	4
97-44	0	172	38.9	?	0	0	0	0	H	4
97-48	0	185	38.7	0	0	0	0	0	S	4

Oct 21/97										
97-12	1	126	39.2	0	0	0	0	0		1
97-14	1	162	39.0	0	0	0	0	0		1
97-21	1	145	38.4	0	0	0	0	0		1
97-24	1	174	38.3	0	0	0	0	0		1
97-26	1	181	38.4	0	0	0	0	0		1
97-33	1	175	38.5	0	0	0	0	0		1
97-34	1	182	39.5	0	0	0	0	0		1
97-39	1	176	38.9	0	0	0	0	0		1
97-42	1	183	38.1	0	0	0	0	0		1
97-47	1	174	37.7	0	0	0	0	0		1
97-11	1	117	38.3	0	0	0	0	0		2
97-17	1	99	38.2	0	0.5	0.5	0	0.5		2
97-18	1	163	38.8	0	0	0	0	0		2
97-29	1	172	38.2	0	0	0	0	0		2
97-32	1	160	38.4	0	0	0	0	0		2
97-35	1	175	38.9	0	0	0	0	0		2
97-40	1	199	38.4	0	0	0	0	0		2
97-45	1	203	38.9	0	0	0	0	0		2
97-49	1	182	39.1	0	0	0	0	0		2
97-10	1	121	38.4	0	0	0	0	0		3
97-16	1	122	39.0	0	0	0	0	0		3
97-20	1	165	38.1	0	0	0	0	0		3
97-22	1	177	38.4	0	0	0	0	0		3
97-27	1	176	39.0	0	0	0	0	0		3
97-30	1	170	38.3	0	0	0	0	0		3
97-36	1	171	38.1	0	0	0	0	0		3
97-38	1	193	38.8	0	0	0	0	0		3
97-43	1	171	38.5	0	0	0	0	0		3
97-46	1	181	37.8	0	0	0	0	0		3
97-13	1	169	38.8	0	0	0	0	0		4
97-15	1	184	38.5	0	0	0	0	0		4
97-19	1	146	38.3	0	0	0	0	0		4
97-25	1	162	38.2	0	0	0	0	0		4
97-28	1	161	38.9	0	0	0	0	0		4
97-31	1	194	36.9	0	0	0	0	0		4
97-37	1	172	38.5	0	0	0	0	0		4
97-41	1	198	38.1	0	0	0	0	0		4
97-44	1	182	38.5	0	0	0	0	0		4
97-48	1	192	38.2	0	0	0	0	0		4

Calf #	Day	Wt (kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Oct 22/97									
97-12	2	126	40.0	0	0	0	0	0	1
97-14	2	161	40.1	0	0	0	0	0	1
97-21	2	144	40.7	0	0	0	0	0	1
97-24	2	173	40.4	0	0	0	0	0	1
97-26	2	178	40.6	0.5	0	0	0	0	1
97-33	2	171	41.2	0	0	0	0	0	1
97-34	2	179	40.5	0	0	0	0	0	1
97-39	2	175	40.9	0	0	0	0	0	1
97-42	2	181	40.8	0	0	0	0	0	1
97-47	2	170	41.1	0	0	0	0	0	1
97-11	2	113	40.0	0	0	0	0	0	2
97-17	2	93	39.1	0	0.5	0.5	0	0.5	2
97-18	2	157	40.6	0	0	0	0	0	2
97-29	2	167	40.6	0.5	0	0	0	0	2
97-32	2	155	41.4	0	0	0	0	0	2
97-35	2	171	41.3	0	0	0	0	0	2
97-40	2	198	39.6	0	0	0	0	0	2
97-45	2	197	40.4	0	0	0	0	0	2
97-49	2	181	39.9	0	0	0	0	0	2
97-10	2	122	39.4	0	0	0	0	0	3
97-16	2	120	40.7	0	0	0	0	0	3
97-20	2	161	40.5	0	0	0	0	0	3
97-22	2	174	41.4	0.5	0	0	0	0	3
97-27	2	174	40.8	0	0	0	0	0	3
97-30	2	172	40.6	0	0	0	0	0	3
97-36	2	174	40.3	0	0	0	0	0	3
97-38	2	190	40.7	0	0	0	0	0	3
97-43	2	174	40.5	0	0	0	0	0	3
97-46	2	177	40.9	0	0	0	0	0	3
97-13	2	164	39.8	0	0	0	0	0	4
97-15	2	176	40.6	0	0	0	0	0	4
97-19	2	141	40.8	0	0	0	0	0	4
97-25	2	157	39.8	0	0	0	0	0	4
97-28	2	159	40.4	0	0	0	0	0	4
97-31	2	189	39.5	0	0	0	0	0	4
97-37	2	173	40.6	0	0	0	0	0	4
97-41	2	189	40.6	0	0	0	0	0	4
97-44	2	176	40.8	0	0	0	0	0	4
97-48	2	191	40.6	0	0	0	0	0	4

Calf #	Day	Wt (kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Oct 23/97									
97-12	3	123	40.9	0	0	0	0	0	1
97-14	3	161	40.5	1.0	0	0	0	0	1
97-21	3	140	39.6	1.0	0	0	0	0.5	1
97-24	3	170	40.6	0.5	0	0	0	0.5	1
97-26	3	175	40.2	0.5	0	0	0	0	1
97-33	3	167	41.0	0	0	0	0	0.5	1
97-34	3	178	40.4	1.0	0	0	0	0.5	1
97-39	3	173	39.8	0.5	0	0	0	0	1
97-42	3	180	39.8	1.0	0	0	0	0.5	1
97-47	3	168	39.9	0.5	0	0	0	0.5	1
97-11	3	112	40.0	0	0	0	0	0	2
97-17	3	91	39.4	1.0	0	0	0	0	2
97-18	3	155	40.4	1.0	0	0	0	0.5	2
97-29	3	167	39.6	1.0	0	0	0	0	2
97-32	3	154	40.4	0.5	0	0	0	0.5	2
97-35	3	175	39.3	0.5	0	0	0	0	2
97-40	3	195	40.3	1.0	0	0	0	0	2
97-45	3	199	40.7	0	0	0	0	0	2
97-49	3	180	40.5	0	0	0	0	0	2
97-10	3	122	39.4	0	0	0	0	0	3
97-16	3	118	40.4	1.0	0	0	0	0	3
97-20	3	160	40.2	1.0	0	0	0	0.5	3
97-22	3	171	40.2	1.0	0	0	0	0	3
97-27	3	176	41.1	1.0	0	0	0	0.5	3
97-30	3	169	39.6	1.0	0	0	0	0	3
97-36	3	171	40.5	1.0	0	0	0	0	3
97-38	3	185	41.0	1.0	0	0	0	0.5	3
97-43	3	172	40.5	0.5	0	0	0	0	3
97-46	3	179	40.5	0.5	0	0	0	0.5	3
97-13	3	167	40.2	1.0	0	0	0	0.5	4
97-15	3	176	40.5	0.5	0	0	0	0	4
97-19	3	145	40.0	0.5	0.5	0	0	0.5	4
97-25	3	163	40.0	0	0	0	0	0	4
97-28	3	153	39.9	0.5	0	0	0	0	4
97-31	3	192	39.1	0	0	0	0	0	4
97-37	3	170	40.6	0.5	0	0	0	0	4
97-41	3	189	40.3	1	0	0	0	0.5	4
97-44	3	178	40.8	1.0	0	0	0	0	4
97-48	3	190	40.3	0	0	0	0	0	4

Calf #	Day	Wt (kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Oct 24/97									
97-12	4	124	40.7	1.0	0	0	0	0	1
97-14	4	161	40.5	1.5	0	0	0	0	1
97-21	4	141	40.5	2.0	0	0	0	1.0	1
97-24	4	167	40.4	1.5	0	0	0	0	1
97-26	4	181	40.8	1.5	0	0	0	0	1
97-33	4	162	40.7	2.0	0	0	0	0	1
97-34	4	178	40.4	2.0	0	0	0	1.0	1
97-39	4	168	40.3	2.0	0	0	0	1.0	1
97-42	4	175	40.6	1.5	0	0	0	0	1
97-47	4	167	39.9	0.5	0	0	0	0	1
97-11	4	112	40.3	1.0	0	0	0	0	2
97-17	4	90	40.4	1.5	0.5	0	0	1.0	2
97-18	4	154	40.9	1.0	0	0	0	0	2
97-29	4	163	41.2	2.0	0	0	0	1.0	2
97-32	4	153	40.5	1.5	0	0	0	0	2
97-35	4	173	40.2	1.5	0	0	0	0.5	2
97-40	4	193	40.6	2.0	0	0	0	1.0	2
97-45	4	197	40.1	1.0	0	0	0	0	2
97-49	4	180	40.5	1.0	0	0	0	0	2
97-10	4	120	40.5	2.0	0	0	0	1.0	3
97-16	4	117	40.9	2.0	0	0	0	1.0	3
97-20	4	157	39.9		0	0	0	0	3
97-22	4	171	41.1	1.5	0	0	0	0	3
97-27	4	178	40.4	2.0	0	0	0	1.0	3
97-30	4	164	40.1	2.0	0	0	0	1.0	3
97-36	4	170	40.7	1.5	0	0	0	0	3
97-38	4	188	40.5	1.5	0	0	0	0	3
97-43	4	170	40.8	1.0	0	0	0	0	3
97-46	4	175	40.6	1.0	0	0	0	0	3
97-13	4	166	40.6	1.0	0	0	0	0	4
97-15	4	175	41.6	1.5	0	0	0	1.0	4
97-19	4	141	40.3	1.5	0	0	0	0	4
97-25	4	158	40.7	1.0	0	0	0	1.0	4
97-28	4	148	40.6	2.0	0	0	0	1.0	4
97-31	4	188	40.6	0.5	0	0	0	0	4
97-37	4	167	40.5	1.0	0	0	0	0	4
97-41	4	186	41.1	1.5	0	0	0	0	4
97-44	4	176	40.5	1.5	0	0	0	0	4
97-48	4	191	40.1	2.0	0	0	0	1.0	4

Calf #	Day	Wt (kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Oct 25/97									
97-12	5	119	40.5	2.0	0	0	0	1.0	1
97-14	5	155	40.2	1.5	1	0	0	1.0	1
97-21	5	135	40.3	2.0	2	0	1	2.0	1
97-24	5	163	40.7	2.0	1	0	0	1.0	1
97-26	5	174	40.1	2.0	0	0	0	1.0	1
97-33	5	156	40.7	1.5	1	0	0	1.0	1
97-34	5	172	41.0	2.0	2.0	0	0	2.0	1
97-39	5	164	41.1	2.0	0	0	0	1.0	1
97-42	5	181	40.0	1.0	1	0	0	1.0	1
97-47	5	166	40.5	1.0	1	0	0	1.0	1
97-11	5	108	40.0	2.0	1	0	0	0.5	2
97-17	5	88	40.6	1.5	1	0	0	1.0	2
97-18	5	149	39.5	1.0	2+	2	2	3.0	2
97-29	5	159	41.1	2.0	2.0		1	2.0	2
97-32	5	149	40.7	2.0	2	0	0	1.0	2
97-35	5	171	40.5	1.0	0	0	0	0	2
97-40	5	191	40.7	1.5	1	0	0	1.0	2
97-45	5	190	40.4	3.0	0	0	1	1.0	2
97-49	5	174	40.8	1.5	0	0	0	0	2
97-10	5	119	40.2	2.0	0	0	0	0	3
97-16	5	113	39.7	2.0	1	0	1	1.0	3
97-20	5	154	40.5	2.0	0	0	0	1.0	3
97-22	5	165	40.6	2.0	1	0	0	1.0	3
97-27	5	170	41.2	2.0	1	0	0	1.0	3
97-30	5	159	39.7	2.0	0	0	0	0	3
97-36	5	164	41.1	2.0	1	0	0	1.0	3
97-38	5	184	41.0	1.0	1	0	0	1.0	3
97-43	5	166	41.4	2.0	1	0	0	1.0	3
97-46	5	170	41.0	2.0	1	0	0	1.0	3
97-13	5	159	40.7	2.0	1	0	0	1.0	4
97-15	5	172	41.1	1.5	1	0	0	1.0	4
97-19	5	142	39.6	0.5	1	0	0	1.0	4
97-25	5	155	40.5	1.5	1	0	0	0.5	4
97-28	5	142	34.8	2.0	3	3-Jan	3	3	4
97-31	5	193	39.2	0.5	0	0	0	0	4
97-37	5	165	40.7	1.5	0	0	0	0.5	4
97-41	5	182	40.9	1.0	0	0	0	0	4
97-44	5	171	40.5	1.0	1	0	0	0.5	4
97-48	5	186	40.8	2.0	1	0	0	1.0	4

Calf #	Day	Wt (kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Note: A large portion of the group are "ill" on first impression.									
Oct 26/97									
97-12	6	no data	no data	3.0	4	4	4	4.0	1
97-14	6	153	39.0	1.0	2	1	0	2.0	1
97-21	6	no data	no data	no data	no data	no data	no data	no data	1
97-24	6	no data	no data	no data	4	4	4	4.0	1
97-26	6	175	39.8	2.0	0	0	0	1.0	1
97-33	6	156	41.3	2.0	1	0	0	1.0	1
97-34	6	no data	no data	no data	no data	no data	no data	no data	1
97-39	6	161	36.9	2.0	2	2	3	3.0	1
97-42	6	176	39.3	1.0	1	0	0	0.5	1
97-47	6	165	40.6	1.5	0	0	0	1.0	1
97-11	6	108	40.3	1.0	1	0	0	1.0	2
97-17	6	88	40.2	2.0	2	1	0	2.0	2
97-18	6	no data	no data	no data	no data	no data	no data	no data	2
97-29	6	157	40.5	2.0	2	0	1	2.0	2
97-32	6	no data	no data	no data	4	4	4	4.0	2
97-35	6	168	40.1	0.5	1			0.5	2
97-40	6	187	39.9	1.0	1	0	0	0.5	2
97-45	6	185	40.7	0.5	0	0	0	1.0	2
97-49	6	no data	no data	no data	4	4	4	4.0	2
97-10	6	116	40.8	2.0	1	0	0	1.0	3
97-16	6	no data	no data	no data	no data	no data	no data	no data	3
97-20	6	154	41.4	1.0	1	0	0	1.0	3
97-22	6	166	41.1	2.0	2	1	0	2.0	3
97-27	6	166	40.8	2.0	2	1	2	2.0	3
97-30	6	160	39.2	2.0	0	0	0	0.0	3
97-36	6	165	40.9	2.0				1.0	3
97-38	6	176	40.7	2.0	2			2.0	3
97-43	6	166	41.0	1.0	1	0	0	1.0	3
97-46	6	170	39.8	2.0	0	0	0	0.0	3
97-13	6	no data	40.1	no data	1	1	3	3.0	4
97-15	6	no data	36.0	no data	2	3	2	4.0	4
97-19	6	143	39.8	2.0	1		1	1.0	4
97-25	6	153	41.0	2.0	1	0	0	1.0	4
97-31	6	189	38.4	?	0	0	0	0.0	4
97-37	6	160	40.8	2+				1.0	4
97-41	6	177	40.2	2.0	1	1	0	2.0	4
97-44	6	167	40.2	2.0	0	0	0	1.0	4
97-48	6	190	40.5	2.0	0	0	0	1.0	4

Calf #	Day	Wt(kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Oct 27/97									
97-14	7	no data	no data	no data	3	3	2	3.0	1
97-26	7	173	40.0	2.0	0	0	1	1.0	1
97-33	7	no data	no data	no data	3	3	2	3.0	1
97-42	7	175	40.1	0.5	1	1	0	1.0	1
97-47	7	164	40.7	2.0	1	0	0	1.0	1
97-11	7	106	39.7	2.0	1	1	0	1.0	2
97-17	7	88	38.9	2.0	2	2	0	1.0	2
97-29	7	161	39.8	2.0	2	2	1	2.0	2
97-35	7	170	40.0	0.5	1	1	0	0.5	2
97-40	7	185	40.4	2.0	1	0	0	1.0	2
97-45	7	185	40.7	2+	2	2	0	1.0	2
97-10	7	117	40.6	2.0	1	1	0	1.0	3
97-20	7	154	40.6	2.0	2	2	2	2.0	3
97-22	7	no data	no data	no data	no data	no data	no data	no data	3
97-27	7	no data	no data	no data	no data	no data	no data	no data	3
97-30	7	161	39.5	0.5	1	1	0	0.5	3
97-36	7	161	40.4	2.0				1.0	3
97-38	7	176	40.7	2.0	1	1	0	1.0	3
97-43	7	168	39.7	0.5	1	0	0	0.5	3
97-46	7	171	40.0	2.0	1	1	0	1.0	3
97-19	7	144	39.1	2.0	1	1	0	1.0	4
97-25	7	150	40.4	2.0	1	0	0	1.0	4
97-31	7	192	38.6	0.0	0	0	0	0.0	4
97-37	7	no data	no data	no data	no data	no data	no data	no data	4
97-41	7	175	39.3	1.5	1	1	0	0.5	4
97-44	7	167	38.9	0.5	1	0	0	0.5	4
97-48	7	187	40.3	2.0	1	0	0	1.0	4

Calf #	Day	Wt(kg)	Temp	Nasal	Dep	Str	Resp	Sick	Group
Oct 28/97									
97-26	8	175	38.2	2.5	0	0	0	0.5	1
97-42	8	172	38.3	0.0	0	0	0	0.0	1
97-47	8	159	39.9	1.5	0	0	0	0.0	1
97-11	8	105	38.6	2.0	1	0	0	1.0	2
97-17	8	86	36.7	3.5	3	2	0	3.0	2
97-29	8	158	40.1	3.0	2		0	2.0	2
97-35	8	166	39.2	0.5	1	0	0	0.5	2
97-40	8	184	39.8	0.0	1	0	0	0.0	2
97-45	8	179	40.8	1.0	2	0	1	1.0	2
97-10	8	114	39.0	2.5	0	0	0	0.5	3
97-20	8	no data	no data	no data	no data	no data	no data	no data	3
97-30	8	157	39.6	0.5	0.5	0	0	0.5	3
97-36	8	156	39.4	1.5	1	0	0	0.5	3
97-38	8	173	41.1	1.5	1.5	0	0	1.0	3
97-43	8	165	40.0	0.0	0.5	0	0	0.5	3
97-46	8	168	39.6	2.0	1	0	0	0.5	3
97-19	8	143	38.5	1.0	0	0	0	0.0	4
97-25	8	146	40.1	1.5	1	0	0	0.5	4
97-31	8	183	38.0	0.0	0	0	0	0.0	4
97-41	8	172	40.2	3.0	1.5	0	0	1.0	4
97-44	8	166	39.8	0.0	0	0	0	0.0	4
97-48	8	181	40.2	2.0	1	0	0	1.0	4